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Ubiquitous Presence of E6 and E7 Transcripts in Human Papillomavirus—Positive Cervical Carcinomas Regardless of Its Type

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The presence of human papillomavirus (HPV) DNA in almost all of the cervical carcinomas is one of the most compelling evidence for the viral carcinogenesis. HPVs are thought to induce cervical carcinoma most likely through the expression of E6 and E7 genes presumably by inactivating the tumor suppressor proteins, p53 and pRb, respectively. Thus far, the presence of HPV E6 and E7 transcripts have been identified only in cervical carcinoma-derived cell lines harboring type 16 or 18, and in a limited number of cervical neoplasia specimens positive for type 16, 18, 33 or 51. To see whether the expression of E6 and E7 genes is an essential finding in HPV-positive cervical carcinoma and cervical intraepithelial neoplasia (CIN), we constructed a reverse transcription-polymerase chain reaction (RT-PCR) assay using a pair of consensus primers in the E6 and E7 regions. Using the assay, E6 transcripts (full-length E6/E7 transcripts) and E7 transcripts (spliced E6/E7 transcripts, E6* mRNA) were identified in 97% (30/31) and 100% (all 31) of cervical carcinomas and in 100% (all 23) and 74% (17/23) of CINs, respectively. This assay also revealed unknown splice donor and acceptor sites of E6* mRNA of less frequent HPV types 31, 35, 52, 56, 58 and 59 based on sequence analyses of the PCR products. Thus, the present study demonstrates that E6 and E7 transcripts of HPV exist in virtually all HPV-positive cervical neoplasia specimens except for the absence of E7 transcripts in some of CINs. ***J. Med. Virol. 62:251–256, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: HPV; RT-PCR; consensus primers; cervical neoplasia; E6/E7 transcripts

INTRODUCTION

Subsets of human papillomavirus (HPV) are thought to be causative agents of cervical carcinomas. A multitude of HPV subtypes including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 70 have been detected in over 90% of invasive cervical carcinomas and the precursor lesions, cervical intraepithelial neoplasias (CINs), by using consensus primer-mediated polymerase chain reaction (PCR) assays [Yoshikawa et al., 1991; Bosch et al., 1995; Rho et al., 1994]. HPV types 16 and 18 have been found in 50–70% of cervical carcinomas, whereas other oncogenic HPV types have been detected in 25–40% [Yoshikawa et al., 1991; Bosch et al, 1995; Nakagawa et al., 1996].

The E6 and E7 genes of HPV 16 and 18 are expressed in cervical carcinoma-derived cell lines tested [Smotkin and Wettstein, 1986], though the expression of these genes has been thus far demonstrated in a limited number of cervical carcinoma specimens [Smotkin et al., 1989; Sherman et al., 1992]. Synergetic actions of E6 and E7 proteins are necessary for immortalization of primary human keratinocytes [Munger et al, 1989a]. It has been suggested that inactivation of tumor suppressor proteins seems to be a central mechanism where by HPV E6 and E7 proteins act as oncoproteins [Dyson et al., 1989; Munger et al., 1989a; Scheffner et al., 1990; Huibregtse et al., 1991; Nevins, 1992; Nakagawa et al., 1995]. More specifically, E6 protein induces degradation of p53 tumor suppressor protein via ubiquitin mediated pathway in cooperation with the E6 AP [Scheffner et al., 1990; Huibregtse et al., 1991]. E7 protein binds to the underphosphorylated form of pRb and inactivates its cell cycle inhibitory function [Dyson et al., 1989; Munger et al., 1989b, 1992]. The phosphorylated pRb releases E2F transcription factor from pRb-E2F complex [Nevins, 1992].

Transcriptional mechanism of E6 and E7 genes has been investigated extensively using HPV types 16 and 18 [Smotkin et al., 1986, 1989]. HPV E6 and E7 genes

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are transcribed from the common promoter p97. The full-length E6/E7 transcripts encode the whole E6 proteins and the shortened E6/E7 transcripts (E6* mRNA) by alternative splicing in the E6 open reading frame (ORF) encode the whole E7 proteins [Smotkin et al., 1989]. Although E6* mRNA encodes the E6* portion as well, the function of the E6* portion in the carcinogenesis of the cervix is not well understood at present [Pim and Banks, 1999]. In this study, we examine the expression of the whole E6 and the whole E7 proteins. We use the word "E7mRNA" or "E7 transcript" as synonymous with "E6* mRNA". For HPV16, the longer and shorter E7 mRNAs (E6* mRNAs) are transcribed with a common splice donor site (nt 226) and two different splice acceptor sites (nt 409, 526). The longer E7 mRNA, named E6*1 mRNA, is predominant over the shorter one, E6*2 mRNA, in both cervical cancer and CIN specimens. Regarding HPV18 mRNA, only one size of E6* mRNA is thus far known [Czegledy et al., 1994].

Several reports have appeared in literature to demonstrate the presence of the E6 and E7 transcripts in cervical carcinoma tissues, positive for HPV types 16, 18, 31, and 51 [Cole and Streeck, 1986; Lungu et al., 1991; Snijders et al., 1992]. The number of HPV types thus far examined are small, however, leaving unanswered the question whether E6 and E7 transcripts are ubiquitously present in all HPV-positive cervical carcinoma specimens. In this study, we sought to examine whether the E6 and E7 transcripts are present in all HPV-positive cervical carcinomas harboring various HPV types using consensus primer-mediated reverse transcription (RT)-PCR assay. We further analyzed the presence of E6 and E7 transcripts in CINs and the splicing pattern of E6* mRNA of a multitude of oncogenic HPVs in cervical carcinomas and CINs.

PATIENTS AND METHODS

Tissues Samples

Tumor specimens were obtained from consenting Japanese women who had given informed consent to use them for research purposes. These women had undergone biopsy or surgery for invasive cervical carcinoma and CIN at the University of Tokyo Hospital and its affiliated hospitals between 1996 and 1997. The specimens included 34 cervical carcinomas and 25 CINs. We excluded HPV-negative specimens from the analysis, thus selecting 31 HPV-positive cervical carcinomas and 23 HPV-positive CINs for the subsequent study. The biopsied tissue samples were snap-frozen in liquid nitrogen or frozen at −80°C immediately after excision. Cryostat sections were cut for haematoxylin and eosin staining to confirm the spread of the lesion. Tissues that neoplastic cells comprised more than 70% of the total cells were used for RNA and DNA extraction. Of the 31 invasive carcinoma patients, 26 had squamous cell carcinoma (SSC), 3 had adenocarcinoma (AC) and 2 had adenosquamous carcinoma (ASC). Of the 23 patients with CIN, 4 had low-grade CIN (CIN I) and 19 had high-grade CIN (CIN II; 5 and CIN III; 14).

Detection and Typing of HPV DNA

The presence and type of HPV were determined by a PCR-based assay (L1-PCR) described by Yoshikawa et al. [1991], with slight modification [Nakagawa et al., 1996]. The L1 region was amplified in 40 PCR cycles of 1.5 min at 95°C, 1.5 min at 48°C, and 2 min at 70°C, using consensus L1 primers L1C1 (5′-CGTAAACGTTTTCCCTATTTTTTT-3′, 1μM), L1C2 (5′-TACCCTAAATACTCTGTATTG-3′, 0.5 μM), and L1C2M (5′TACCCTAAATACCCTATATTG-3′, 0.5 μM). Each reaction product (10 μl) was electrophoresed on a 4% agarose gel, stained with ethidium bromide, and viewed under UV light. HPV types were identified on the basis of restriction fragment length polymorphism (RFLP). Initial typing of amplified HPV DNA fragments were performed by digestions with DdeI and RsaI, and then confirmed by digestions with at least three enzymes selected from AccI, AluI, BstXI, FokI, HaeIII, HinfI, KpnI, MaeI, MaeIII, PstI and XbaI. The assay can type at least 26 genital HPVs (types 6, 11, 16, 18, 30, 31, 33–35, 39, 42–45, 51, 52–56, 58, 59, 61, 66, 68, and 70) [Yoshikawa et al., 1991; Nagano et al., 1996].

HPV types identified in 31 cervical carcinomas were as follows; HPV 16 in 13, HPV 18 in 8, HPV 31 in 2, HPV 33 in 3, HPV 35 in 1, HPV 52 in 2 and HPV 58 in 2. HPV types detected in 23 CINs were as follows; HPV16 in 8, HPV 31 in 1, HPV 33 in 3, HPV35 in 1, HPV 51 in 1, HPV 52 in 2, HPV 56 in 2, HPV 58 in 4 and HPV 59 in 1.

RNA Isolation and RT

Total RNA was extracted from tumor specimens using the RNeasy Mini Kit (QIAGEN, Inc. Chatsworth, CA). From 20 μg of total RNA, mRNA was isolated by the Oligotex-dT Super (TaKaRa BIOMEDICALS, Japan). For the RT-PCR, total volume (10 μl) of mRNA preheated at 65°C for 5 min served as a template for single-strand cDNA synthesis in a 20 μl reaction mixture containing 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 0.5 mM dNTPs, 200 μM oligo (dT) primer, 20 units of RNase inhibitor, and 200 units of M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) at 37°C for 60 min. The reaction was terminated at 95°C for 3 min.

Design of Consensus Primers for E6 and E7 mRNA Expression Analysis

We used the known conserved sequences among multiple oncogenic HPV types in E6 ORF and in E7 ORF as 5′ primer and 3′ primer of the RT-PCR assay, respectively. E6C1 (5′-ACCGAAAACGGTTGAACCGAAAACGGT-3′), previously described by us [Yoshikawa et al., 1990], was used as the 5′ consensus primer (the corresponding base position in HPV16; nt 35–61) and pU-2R (5′-CTCGACAGCGAATTAACGAG-3′), described by Fujinaga et al. [1991], was used as 3′ consensus primer (the corresponding base position in HPV16; nt 637–656), respectively. Both consensus primers maintained

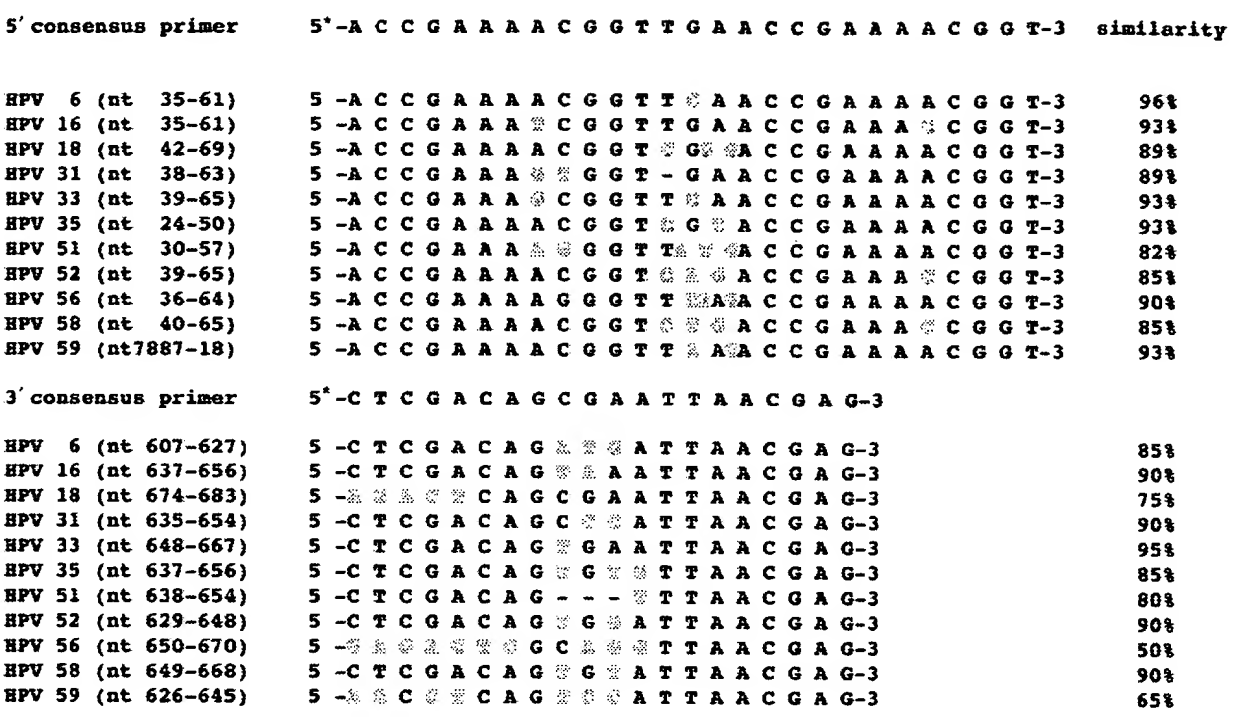

Fig. 1. Sequence comparison of the consensus primers (E6C1 and pU-2R) with various HPV types. For each HPV type, the nucleotides mismatched with the consensus sequences are shown by outlined letters and absence of the corresponding nucleotide is indicated by "-".

around 80–90% homology among multiple oncogenic HPVs such as types 16, 31, 33, 35, 39, 45, 51, 52, 58, 59 and 70, although they are not identical to any of these HPV types (Fig. 1). At first, the PCR assay was confirmed to be able to detect the cloned DNA of at least 13 oncogenic genital HPVs such as types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 70 sensitively as the PCR assay with specific primers except for types 56 and 59, that were shown a slight decrease of sensitivity (data not shown). The expected lengths of the RT-PCR products are 621bp for full-length E6/E7 transcripts (E6 transcripts), 440bp for E6*1 transcripts (E7 transcripts) and 323bp for E6*2 transcripts (E7 transcripts) in HPV16.

RT-PCR and cDNA Sequencing Method

RT-PCR was performed using the consensus primers for E6 and E7 genes, E6C1 and pU-2R. Each reaction mixture consisted of 4 μl of cDNA, 0.8 μM of each primer, 50 μM of each dNTPs, 1× PCR buffer, and 1 unit of the Ampli Taq Gold (Perkin-Elmer Applied Biosystems) to a final volume of 25 μl. The reaction mixture was subjected to 35 cycles of 0.5 min at 95°C, 0.5 min at 48°C, and 1 min at 70°C after the hot start of 7 min at 95°C (in Perkin-Elmer thermal cycler model 2400). Two μl of the PCR products were electrophoresed on 2% agarose gels (NuSieve, FMC Bioproducts, Rockland, ME). Bands were cut from agarose gels, and DNA was extracted using the SUPREC-01 (TaKaRa BIOMEDICALS, Japan). DNA was sequenced directly using 5' consensus and 3' consensus primers (E6C1 and pU-2R), by the Taq DyeDeoxy terminator cycle sequencing reaction for sequence analysis on the Applied Biosystems model 373A.

RESULTS

The RT-PCR assay used in the present study was able to detect E6 mRNA (E6 transcripts) and E6*

TABLE I. Detection of HPV E6 and E7 Transcripts in HPV-Positive Cervical Neoplasias

Lesion	HPV type 16	18	31	33	35	51	52	56	58	59	E6 transcript (%)	E7 transcript (%)
CIN I	1*							1	1*	1	4/4 (100%)	2/4 (50%)
CIN II	1*			1	1*			1	1		5/5 (100%)	3/5 (60%)
CIN III	6†		1*	2		1	2		2		14/14 (100%)	12/14 (86%)
Invasive cancer	13	8#	2	3	1		2		2		30/31 (97%)	31/31 (100%)

*Only E6 transcript was detected in each sample.
†Only E6 transcript was detected in one of 6 samples.
#Only E7 transcript was detected in one of 8 samples.

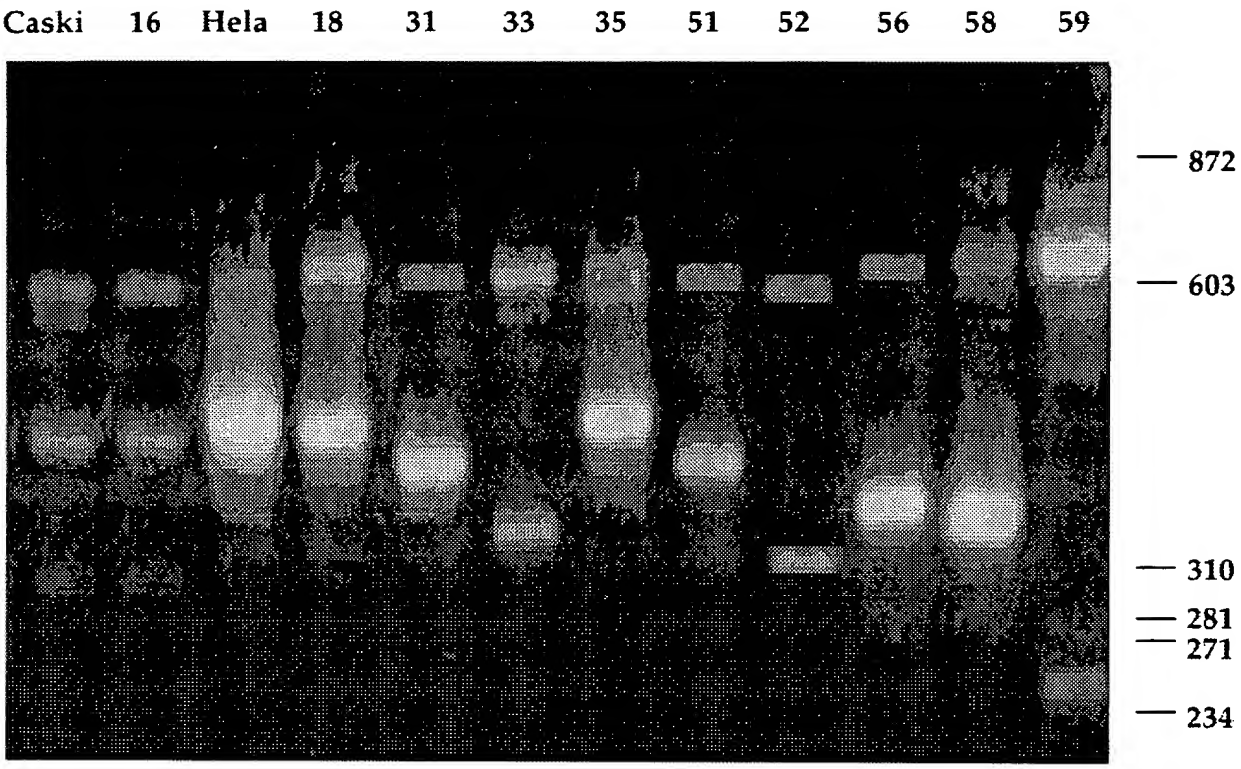

Fig. 2. Detection of E6 and E6* mRNAs (E6 and E7 transcripts) in cervical neoplasia samples and cervical cancer-derived cell lines. The figures correspond to HPV types. The upper band of each lane is amplified from the full-length E6/E7 cDNA and the lower bands are amplified from the spliced E6/E7 cDNA. In lanes for HPV 16-positive Caski cells and carcinoma sample, the middle and the lowest bands correspond to the E6*1 and E6*2 cDNA, respectively. The positions of molecular weight markers (in base pairs) are shown on the right of the panel.

mRNA (E7 transcripts) in cervical cancer and CIN specimens positive for HPV types 16, 18, 31, 33, 35, 51, 52, 56, 58 and 59 (Table I). The E6 and E7 transcripts were identified in 30 (97%) of the 31 and all the 31 (100%) HPV-positive cervical carcinomas, respectively. As for the full length E6/E7 PCR product (E6 mRNA), to avoid the amplification from residual HPV DNA in RNA samples, we used mRNA samples purified from total RNA samples using the Oligotex-super beads for PCR amplification. In addition, it was confirmed that the full length E6/E7 genes was not amplified directly from the mRNA samples before RT reaction (data not shown), indicating that the full length E6/E7 bands were not amplified from residual HPV DNA, but from the cDNAs produced after RT reaction. Thus, the present data can be interpreted to provide evidence for the presence of human papillomavirus E6 and E7 transcripts in almost all HPV DNA-positive cervical carcinomas. On the other hand, the E6 and E7 transcripts were found in the 23 (100%) CINs and 17 (74%) of the 23 CINs, respectively. It seems that E7 transcripts exist in a majority, but not all CIN lesions, whereas E6 transcripts are present in almost all of the CIN lesions. Based on Barbosa and Wettstein's data, it is unlikely that E7 protein is present in samples where only the E6 transcript (unspliced E6–E7 transcript) is detected [Barbosa and Wettstein, 1988].

Splicing patterns of E6* mRNA detected in cervical neoplasias with HPV types 16 and 18 were identical to those found in Caski and HeLa, cervical cancer-derived cell lines, respectively (Fig. 2). The patterns also agreed with those previously detected in cervical carcinoma specimens [Smotkin et al., 1989; Czegledy et al., 1994]. Thus, it appears that the RT-PCR assay using consensus primers is able to detect HPV16 and 18 E6* mRNA accurately. There was only one size of E6* mRNA in cervical neoplasia harboring oncogenic HPVs other than HPV16, though longer and shorter E6* (E6*1 and E6*2) mRNAs were found in HPV16-positive neoplasia specimens at a considerable rates; 100% [13/13] vs. 77% [10/13] in invasive carcinomas and 63% [5/8] vs. 50% [4/8] in CINs, respectively.

DISCUSSION

The expression of HPV16 E6 and E7 genes was initially investigated by using Northern blot analysis and detected only in less than 30% of HPV16-positive cervical carcinoma and CIN specimens [Lehn et al., 1985; Shirasawa et al., 1988]. Recently, the detection rate of E6 mRNA and E6* mRNA of HPV16 and 18 was improved using type-specific RT-PCR to the level of around 80–90% [Hsu et al., 1993; Czegledy et al., 1994, 1995; Selinka et al., 1998]. Our consensus primer-mediated RT-PCR assay seems to be as sensitive as

TABLE II. E6* Splice Sites in Various HPV Types and Size of RT-PCR Product

HPV type	Donor site	Acceptor site	PCR product size E6* cDNA (bp)
	A ↓ A	CCCCC C ↓G	
Consensus	GAGGTGAGT	TTTTTTNTAGGT	
	226 ↓	409 ↓	
HPV16	GAGGTATAT	TGTTAATTAGGT	440
	226 ↓	526 ↓	
	GAGGTATAT	CTTGTTGCAGAT	323
	233 ↓	416 ↓	
HPV18	GAGGTATTT	TATTAATAAGGT	460
	210 ↓	413 ↓	
HPV31	AAGGTCAGT	TGTTAATTAGGT	415
	(GAGGTATTA)		
	231 ↑		
	231 ↓	509 ↓	
HPV33	GAGGTATAT	CGTTGGGCAGGG	352
		(TATTAATTAGGT)	
		414 ↑	
	232 ↓	415 ↓	
HPV35	GAGGTATAT	TATTAATTAGGT	451
	177 ↓	402 ↓	
HPV51	CAGGTAGTG	TATCGATAAGGT	401
	224 ↓	502 ↓	
HPV52	GAGGTATAC	CGTTGGACAGGG	333
		(TAACTATTAGAT)	
		407 ↑	
	161 ↓	416 ↓	
HPV56	GAGGTATTA	TATTAATAAGGT	381
	232 ↓	510 ↓	
HPV58	GAGGTATAT	CGTTGGACAGGG	352
	183 ↓	582 ↓	
HPV59	GAGGTATTT	ATTGTTTTAGAT	258

The required GT and AG sequences for splicing are underlined.
Arrows indicate splice junction sites.
The identical nucleotides to the consensus sequences are expressed in boldface.
The previously reported splicing sites deduced only based on sequence comparison are shown in the parentheses.

type-specific RT-PCR for the detection of the HPV16 and 18 mRNA [Czegledy et al., 1994; Selinka et al., 1998]. The higher detection rate in the present study may be due to use of pure mRNA samples. In addition, the RT-PCR assay enabled us to detect E6 and E7 transcripts of a broad spectrum of oncogenic HPVs in a sensitive way. The RT-PCR assay using a pair of consensus primers was shown to be a simple and inexpensive method compared with type-specific RT-PCRs for oncogenic HPVs.

The splice donor and acceptor sites of E6* mRNA for HPV types 16, 18 and 51 were shown to be identical to the previously reported sites based on sequence analyses of the transcripts (Table II). The splice donor and acceptor sites of E6* mRNA for HPV types 31, 52 and 56 have been deduced based on the consensus sequence similar to the splice donor and acceptor sites of HPV types 16 and 18 [Goldsborough et al., 1989]. The RT-PCR assay identified the actual splice donor and acceptor sites of E6* mRNA of these HPV types in primary cervical carcinomas and CINs (Table II). Although the actual splice donor and acceptor sites of HPV 56 were identical to those deduced from the consensus sequence, the splice donor site of HPV type 31 (nt 210) and the splice acceptor sites of HPV types 33 (nt 509) and 52 (nt 502) were different from the deduced splice donor sites of HPV type 31 (nt 231) and acceptor sites of HPV types 33 (nt 414) and 52 (nt 407) [Goldsborough et al., 1989], respectively. These newly identified splice sites had the consensus sequences whose nucleotide positions were different from the deduced sites. In addition, the RT-PCR assays also identified unknown splice donor and acceptor sites of E6* mRNA for HPV types 35, 58 and 59 in keeping with the consensus sequences.

Though the incidence of E6 mRNA in CIN was similar to that in cervical carcinomas; 100% (23/23) vs. 97% (30/31), the detection rate of E6* mRNA (E7 transcripts) increased with progression of the disease from low-grade CIN to invasive carcinoma; 50% (2/4) in CIN I, 60% (3/5) in CIN II, 86% (12/14) in CIN III, and 100% (31/31) in cervical carcinomas (Table I). These findings are in accord with the data that detection rates of E6*1 and E6*2 mRNAs in HPV16-positive neoplasia increase with disease progression from low-grade CIN to invasive carcinoma [Sotlar et al., 1998]. Therefore, monitoring of E6* mRNA expression using RT-PCR assay employed here could be a useful predictor for progression of CIN irrespective of HPV types.

In conclusion, the present study demonstrates that E6 and E7 transcripts of HPV exist in virtually all of the HPV-positive cervical carcinoma specimens. Con-

cerning HPV-positive CIN, though E6 transcripts were found in all CINs, E7 transcripts were not detected in some of CINs and the incidence increases with disease progression. Hence, the E7 expression may be not crucial for the development of CIN, but a prerequisite for the progression of CIN to develop cervical carcinoma, irrespective of HPV types. Furthermore, splicing mechanism in synthesizing E6*mRNA for a variety of oncogenic HPVs other than types 16 and 18 was similar to that for types 16 and 18. In view of the ubiquitous presence of E6 and E7 transcripts and their common splicing patterns, a multitude of oncogenic HPVs including types 16 and 18 seem to be involved in a similar way during the development of cervical carcinoma.

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